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A Role for Pref-1 and HES-1 in Thymocyte Development

Midori Kaneta,* Masatake Osawa,† Mitsujiro Osawa,* Kazuhiro Sudo,* Hiromitsu Nakauchi,§ and Yousuke Takahama2¶

T lymphocyte development requires a series of interactions between developing thymocytes and thymic epithelial (TE) cells. In this paper we show that TE cells in the developing thymus express Pref-1, a Delta-like cell-surface molecule. In fetal thymus organ cultures (FTOC), thymocyte cellularity was increased by the exogenous dimeric Pref-1 fusion protein, but was reduced by the soluble Pref-1 monomer or anti-Pref-1 Ab. Dimeric Pref-1 in FTOC also increased thymocyte expression of the HES-1 transcription factor. Thymocyte cellularity was increased in FTOC repopulated with immature thymocytes overexpressing HES-1, whereas FTOC from HES-1-deficient mice were hypocellular and unresponsive to the Pref-1 dimer. We detected no effects of either Pref-1 or HES-1 on developmental choice among thymocyte lineages. These results indicate that Pref-1 expressed by TE cells and HES-1 expressed by thymocytes are critically involved in supporting thymocyte cellularity. *The Journal of Immunology, 2000, 164: 256–264.

Most T lymphocytes are generated in the thymus. Several molecular interactions between developing T cells and thymic stromal cells have been shown to be critically involved in T cell development. Such interactions include IL-7 signals (1), E-cadherin-mediated interactions (2), and TCR recognition of peptide-loaded MHC molecules (3). However, in vitro attempts to reconstitute a functional thymic environment has been successful only in organ-culture conditions in the presence of thymic stromal cells. Thus, it is likely that uncharacterized molecular interactions between thymocytes and stromal cells play essential roles in the thymic phase of T lymphocyte development.

With the aim of identifying new regulators of thymocyte development, we have found that Pref-1 is highly expressed by thymic stromal cells during early ontogeny. Pref-1 (4), also known as dlk (delta-like) (5), FA1 (fetal Ag 1) (6), or SCP-1 (stromal cell-derived protein 1) (7), is a Delta-like cell-surface transmembrane protein containing six tandem epidermal growth factor-like repeats, which are highly homologous to Delta/Notch-family proteins including Delta and Notch, that are involved in Drosophila neural development (8). Unlike other Delta-family molecules, Pref-1 lacks the DSL motif, which is suggested to be crucial for the interaction with Notch-family molecules (9), suggesting that Pref-1 may exert its biological function(s) independent of Notch-family receptors or their downstream signals, including the expression of HES family of basic helix-loop-helix transcription factors (10). Although the role of Pref-1 in thymocyte development was unknown, stromal cell expression of Pref-1 has been shown to play an important role in regulating the development and growth of adjacent cells; i.e., inhibiting adipocyte differentiation (4), promoting the proliferation of hematopoietic stem cells (11), and modulating IL-7 dependency of pre-B cell proliferation (12).

The present study shows that Pref-1 signals in the thymus regulate the cellularity of developing thymocytes. Despite previous assumptions based on the lack of the DSL motif, Pref-1 markedly increased immature thymocyte expression of the HES-1 transcription factor. Our data lend support to the notion that Pref-1 plays a role in thymocyte development by regulating thymocyte levels of HES-1 expression.

Materials and Methods

Mice

HES-1-deficient mice (13) were provided by Dr. Ryoichiro Kageyama (Institute for Viral Research, Kyoto University, Kyoto, Japan). Because HES-1-/- mice were embryonic lethal, HES-1+/+ fetuses were obtained by crossing HES-1+/+ mice. Day 14 embryos were screened for HES-1 genotype as previously described (13). C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan).

Recombinant Pref-1 proteins

For monomeric recombinant soluble protein containing the extracellular domain of mouse Pref-1, the Pref-1-His6 protein attached at the C terminus with amino acid sequences corresponding to influenza hemagglutinin (HA) epitope and hexahistidine was expressed. For dimeric Pref-1, the Pref-1-Fc fusion protein attached with the Fc portion of human IgG1 was expressed. cDNA encoding extracellular domain of mouse Pref-1 was cloned from NIH-3T3 cells by RT-PCR using the following primers (sense, 5'-CCG AAT TCG AGA TGA TCG CGA CCG GAG CCC TC-3'; and antisense, 5'-TAA GTA GTG GTG AGA AGG GGT GTA CTC TT-3'). The PCR product was inserted into either an EcoRI site of the pSMT-201-HA-His6 vector (14) or a SpeI site of the pSMT-Fc vector (M. Ohashi, unpublished data). Pref-1-HA-His6 or Pref-1-Fc inserts were subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA). CHO-ras clone I cells (provided by Dr. T. Shirahata, Kyoto University, Kyoto, Japan) were transfected with the expression vector and
selected for high expression clones in the presence of 300 μg/ml hygro- mycin. Recombinant Pref-1- HA-His6 protein and Pref-1-Fc protein were purified from CHO cell culture supernatants by affinity chromatography over Hi-Trap Chelating-Sepharose and Hi-Trap Protein G-Sepharose (Am- ersham Pharmacia Biotech, Uppsala, Sweden), respectively.

**Anti-Pref-1 mAb**

A hybridoma clone, 6C, producing a mAb against extracellular region of mouse Pref-1, was established by fusing mouse myeloma cells with spleen cells from Armenian hamsters immunized with the Pref-1-Fc protein. Hy- bridoma cells were initially screened for binding activity to Pref-1 recom- binant proteins and then for staining activity to a stable line of CHO cells that had been transfected with full-length mouse Pref-1 cDNA. mAb 6C purified from culture supernatants over protein G affinity chromatography specifically bound to Pref-1 recombinant proteins but not to other Fc fusion proteins or HA-tagged proteins, and specifically stained Pref-1-transfected CHO cells but not untransfected CHO cells. To analyze the reactivity to membrane-bound form of mouse Pref-1, Pref-1-transfected or untransfected CHO cells were incubated with mAb, followed by staining with FITC-conjugated anti-hamster IgG (PharMingen, San Diego, CA).

**Thymic epithelial (TE) cell lines**

Cortical TE cell lines used were TEP1-1 (15), TEC (16), and 1308.1 (17). FITC-conjugated anti-hamster IgG Ab (PharMingen, San Diego, CA). Anti-Pref-1 mAb was selected for high expression clones in the presence of 300 μg/ml hygromycin B.

**Fetal thymus organ culture (FTOC)**

Day 14 fetal thymus lobes from B6 mice were cultured in organ in the presence or absence of indicated reagents, as described previously (20).

**Multicolor flow cytometry and cell sorting**

Single cell suspensions were stained using mAbs (PharMingen) and ana- lyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA). The purity of CD4+ lymphocytes was measured using the ABI PRISM 7700 analytical thermal cycler (Perkin-Elmer Applied Biosystems). The purity of CD4+ lymphocytes was measured using the ABI PRISM 7700 analytical thermal cycler (Perkin-Elmer Applied Biosystems). Equal numbers (1000–2500/lobe) of sorted GFP+ cells were collected (25). Retrovirus infection into immature thymocytes and the detection of recombinant soluble proteins containing the extracellular region of mouse IL-7 in 96-well flat-bottom culture plates. Virus-infected thymocytes were collected (25). Retrovirus infection into immature thymocytes and the detection of their development on January 21, 2018

**Immunohistochemical staining**

Frozen sections (5 μm) of day 14 fetal, 0-day neonatal, and 7-wk-old adult thymus lobes were incubated with anti-Pref-1 mAb, followed by biotiny- lated anti-hamster IgG Ab and HRP-conjugated streptavidin. The sections from organ-cultured fetal thymus lobes were incubated with anti-HES-1 Ab, followed by HRP-conjugated anti-rabbit IgG Ab.

**Western blotting**

Fetal thymus lobes cultured in organ for 3 days were lysed in a buffer containing 1% Nonidet P-40. The lysates of equal numbers of the cells were electrophoresed on a SDS-polyacrylamide gel, transferred to a nylon membrane, and detected for HES-1 using anti-HES-1 Ab (provided by Dr. T. Sudo, Torai, Japan) followed by HRP-conjugated anti-rabbit IgG Ab and ECL detection reagents (Amersham Pharmacia). Anti-Flag mAb (East- man Kodak, Tokyo, Japan) was used for the detection of Flag-tagged HES-1 proteins. Densitometric analysis was done by electronic scanning of the film followed by the measurement using NIH Image software (version 1.61, http://rsb.info.nih.gov/nih-image/).

**Retrovirus transfer of HES-1 in developing thymocytes**

The pSK plasmid (Stratagene, La Jolla, CA) was modified to contain syn- thetic oligonucleotide corresponding to the Flag peptide (MDYKD- DDDDQ) with a BglII restriction site at the 3′ end. To generate the BglII site at the 5′ end of rat HES-1 cDNA, pCMV-2-HES-1 plasmid (24) was PCR amplified with the following primers (sense, 5′-GGA AGA TCT CCA GCT GAT ATA ATG GAG AAA AAT-3′ and antisense, 5′-AGC GGC GCC AGC TAC TTG CAT TGG TAG CAG TTG AGT AG-3′). The resulting PCR product was cloned into BglII-BamHI sites of the pSK-Flag plasmid. The Flag-HES-1 insert was subcloned into the EcoRI site of the pMSV-IRES-GFP plasmid (25). Retrovirus vector was transfected into GP+E-86 packaging cells, and clones producing <105 CFU/ml were se- lected. Retrovirus infection into immature thymocytes and the detec- tion of their development on January 21, 2018

**Measurement of HES-1 activity by luciferase reporter assay**

293 T cells were transfected with 5 μg of the plasmid containing luciferase reporter gene under the control of the HES-1 promoter (27). Luciferase activity in cell lysates on day 2 was measured as described (27).

**Results**

**Pref-1 expression by TE cells**

To begin analyzing biological function of Pref-1, we first prepared recombinant soluble proteins containing the extracellular region of murine Pref-1, a Pref-1 monomer, and a Pref-1 homodimer fused to Fc region of human IgG1 (Fig. 1A). We also immunized ham- sters with recombinant Pref-1 to generate mAb that specifically reacted with Pref-1 recombinant proteins as well as Pref-1-transfected CHO cells but not with untransfected CHO cells (Fig. 1B).
Immunohistochemical analyses of thymus tissues with this Ab revealed a developmentally regulated pattern of reactivity, with widespread staining of stromal cells in the fetal thymus, and progressive loss of reactivity with increasing age, such that scattered Pref-1+ cells were detected in the adult thymus (Fig. 1, C and D).

To further examine the expression of Pref-1 and related molecules in the thymus, semiquantitative RT-PCR analysis was performed for Delta-family molecules including Pref-1 as well as Notch- and HES-family molecules (Fig. 2). cDNAs from various thymocyte samples were normalized using the real-time quantitative PCR assay for a housekeeping enzyme GAPDH (Fig. 2A), and an equivalent amount of cDNA was employed for further semiquantitative analysis (Fig. 2B). Jagged-1, Jagged-2, Notch-1, Notch-2, Notch-3, Notch-4, HES-1, and HES-5, in addition to another housekeeping enzyme HPRT, were consistently expressed during thymus ontogeny and among adult thymocyte subpopulations (Fig. 2B). The expression of HES-3 was barely detected in the thymus samples (Fig. 2B). In contrast, however, variable expression of Pref-1, DLL-1, and DLL-3 was noticed among thymocyte subpopulations. Pref-1, DLL-1, and DLL-3 were expressed higher in stromal cells than in lymphoid cells within adult thymus (Fig. 2B). Unlike DLL-1 and DLL-3, Pref-1 in the thymus was expressed higher in early ontogeny, consistent with immunohistochemical results (Fig. 1C). More interestingly, within TE cell lines, Pref-1 was expressed only by a group of cortical TE cell lines such as TEP1-1 and TEC but not by other cortical TE cell line such as 1308.1 or by medullary TE cell lines such as TE71 and Z210R (Fig. 2B).

FIGURE 1. Recombinant Pref-1 proteins and anti-Pref-1 Ab. A, SDS-PAGE analysis of recombinant Pref-1-Fc protein and Pref-1 monomer protein. NR, nonreduced; R, reduced. B, mAb 6C binds to Pref-1-transfected CHO cells but not untransfected CHO cells. Also shown is a control histogram of Pref-1-transfected CHO cells stained with normal hamster IgG. C and D, Anti-Pref-1 Ab 6C staining of thymus sections from fetal (days 14 and 16), newborn (0 day old), and adult (7 and 3 wk old) B6 mice. Labeling in the 3-wk-old thymus section is indicated by arrows. Small dots of reaction product represent endogenous peroxidase activity that is also observed in the absence of Ab. Asterisk, capsule; C, cortex; M, medulla. (Bar = 200 μm.) Shown are representative results from more than three independent measurements.

FIGURE 2. RT-PCR analysis for the expression in the thymus of Delta-family molecules including Pref-1 as well as Notch- and HES-family molecules. A, Normalization of cDNAs from various thymocyte samples using quantitative PCR for a housekeeping enzyme GAPDH. Adult thymus was teased on defrosted slide glasses and filtered over nylon mesh to fractionated into filtered thymocytes and filter-trapped stromal cells. Indicated are relative GAPDH levels (means ± SDs) of normalized cDNAs from indicated cells. B, Quantitatively normalized cDNAs as shown in A were employed for further RT-PCR analysis for indicated molecules. PCR for another housekeeping gene, HPRT, was employed to ensure the normalization of cDNA quantity. Shown are representative results from two independent measurements.
These results indicate that Pref-1 is expressed in the thymus at higher levels during fetal development and predominantly by a population of cortical T cells.

**Effects of recombinant Pref-1 and anti-Pref-1 Ab in FTOC**

To examine the function of Pref-1 in T lymphocyte development, thymocyte development was analyzed in FTOC in the presence of recombinant Pref-1 proteins. As shown in Fig. 3A, the Pref-1-Fc dimer increased the numbers of developing thymocytes in a dose-dependent manner, whereas the Pref-1 monomer decreased the cell numbers. Other than the cellularity, however, we detected no alterations in T cell development including CD4/CD8 phenotype and TCR-αβ/TCR-γδ ratio (Fig. 3B). Time course analysis showed that the Pref-1-Fc dimer and the Pref-1 monomer affected thymocyte cellularity within 2–3 days in the culture (Fig. 3C). The effects by Pref-1 recombinant proteins appeared to be mediated by biological functions of Pref-1 proteins, because other Fc-containing proteins such as normal human IgG1 and CTLA-4-Ig fusion protein did not affect the cellularity in parallel cultures (data not shown). The cellularity was not affected by the addition of the Pref-1 dimer in single cell suspension cultures of fetal thymocytes in the absence or presence of IL-7 (Fig. 3D), suggesting that Pref-1 may be capable of supporting thymocytes only in the presence of other factors that are supplied in organ cultures but are not present in suspension cultures.

To test a possible involvement of endogenous Pref-1 expressed in developing thymus, anti-Pref-1 mAb was included in FTOC. As shown in Fig. 4A, anti-Pref-1 Ab decreased the cellularity of thymocytes in the culture without affecting the CD4/CD8 phenotype or TCR-αβ/TCR-γδ ratio (Fig. 4B).

These results indicate that the Pref-1 dimer augments the cellularity of developing thymocytes in FTOC, whereas either the Pref-1 monomer or the monoclonal anti-Pref-1 Ab diminishes the cellularity perhaps by neutralizing endogenous Pref-1. These data suggest that Pref-1 expressed by stromal cells in developing thymus is involved in supporting the cellularity of developing thymocytes.

**Pref-1 affects the expression of HES-1 in developing thymocytes**

To explore molecular mechanism by which Pref-1 regulates thymocyte cellularity, the expression levels of various molecules including transcription factors were examined by semiquantitative RT-PCR for Pref-1-treated thymocytes recovered from FTOC. Consequently, we found that mRNA expression of a basic helix-loop-helix transcription factor HES-1 was up-regulated by Pref-1-treated thymocytes within 3 days in the culture (Fig. 5A). The expression levels of other transcription factors including HES-5, GATA-3, LIF-1, TCF-1, PEBP2αA, and PEBP2αB as well as Notch-family molecules including Notch-1, Notch-2, Notch-3, and Notch-4 were not markedly affected (Fig. 5A and data not shown). Pref-1 treatment indeed up-regulated protein levels of HES-1 in fetal thymocytes (Fig. 5, B and C). Though consistent, the Pref-1-mediated increase in HES-1 protein levels was always modest by the immunoblot analysis (Fig. 5B). We think that this modest increase in total thymocyte lysates reflected the basal expression of HES-1 proteins by many subcapsular thymocytes even in control FTOC without Pref-1 treatment (Fig. 5C, left panel). Although it is still unclear how to reconcile the clear increase in total RNA levels (Fig. 5A) and the modest increase in total protein levels (Fig. 5B), it is important to note that majority of thymocytes in Pref-1-treated
FTOC now expressed markedly higher levels of HES-1 proteins (Fig. 5C). Thus, the addition of Pref-1 in FTOC increases HES-1 expression by developing thymocytes, the increase coincided with the increase in thymocyte cellularity.

Impaired Pref-1 responsiveness and cellularity of HES-1-deficient thymocytes

To directly test whether HES-1 is involved in the effects of Pref-1 onto thymocytes, FTOC was conducted using thymus lobes from HES-1-deficient mice. As has been recently reported, HES-1-deficient mice are embryonic lethal during late stage of fetal development (13). As summarized in Table I, some, but not all, HES-1-deficient fetuses were athymic. Organ culture of HES-1-deficient fetal thymus lobes revealed a markedly reduced cellularity compared with normal littermate controls (Table I and Fig. 6A). Importantly, the addition of a dimeric Pref-1-Fc fusion protein did not significantly increase thymocyte cellularity in HES-1-deficient cultures (Fig. 6A). It should be noted that, other than cellularity, the CD4/CD8 phenotype and the TCR-αβ/TCR-γδ ratio in HES-1-deficient FTOC was virtually normal in the absence or presence of Pref-1 (Table I, Fig. 6R, and data not shown).

Thus, the cellularity of thymocytes and Pref-1-responsiveness were both impaired in HES-1-deficient thymocytes, suggesting that HES-1 is involved in Pref-1-mediated support of thymocyte cellularity.

Retroviral overexpression of HES-1 increases thymocyte cellularity

To further examine the possibility that HES-1 in thymocytes is involved in supporting the cellularity of thymocytes, we examined the consequence of HES-1 overexpression in developing thymocytes using the retrovirus-mediated gene transfer technique (25, 260 REGULATION OF THYMOCYTE DEVELOPMENT BY Pref-1 AND HES-1

FIGURE 4. Effects of anti-Pref-1 Ab in FTOC. Day 14 fetal thymus lobes from B6 mice were cultured in organ for 5 days in the absence or presence of indicated concentrations of either anti-Pref-1 mAb 6C or normal hamster IgG. A, Viable cell numbers per lobe were measured using trypan blue dye exclusion method. Means ± SEs of cell numbers (n ≥ 12) are shown. Asterisks indicate significant (*, p < 0.01; **, p < 0.001 by Student’s t test) differences in cell numbers from control cell numbers in the absence of Ab. The addition of normal hamster IgG did not significantly (N.S.) alter the cell numbers. B, Thymocytes were two-color stained as indicated. Representative results from five (A) and three (B) independent measurements are shown.

FIGURE 5. Pref-1 affects the expression of HES-1 in developing thymocytes. Day 14 fetal thymus lobes from B6 mice were cultured in organ for 3 days in the absence or presence of Pref-1-Fc dimer (10 μg/ml in A, and 80 μg/ml in B and C). A, RT-PCR analysis of cDNAs prepared from Pref-1-treated thymocytes. Note that expression levels for a house-keeping gene β2-microglobulin (β2m) were equivalent between cDNA samples in the absence and presence of Pref-1. B, Western blotting analysis for HES-1 expression in Pref-1-treated thymocytes. Lysates from equal number (4 × 10⁵) of fetal thymocytes cultured in organ for 3 days in the absence or presence of Pref-1 were electrophoresed and immunoblotted for HES-1 using anti-HES-1 Ab. Underlined numbers indicate relative densitometric intensity of the signals. The up-regulation of HES-1 expression was modest, but consistent (n = 4) and specific, as nonspecific signals (>30 kDa) on the same blot gave equivalent intensity of signals between the two lysates. C, Immunohistochemical analysis of HES-1 expression in the sections of fetal thymus lobes cultured for 3 days in the absence or presence of Pref-1. Shown are representative results from three (A), four (B), and three (C) independent experiments.
Table I. Impaired thymus formation and thymocyte development in HES-1-deficient mice

<table>
<thead>
<tr>
<th>Fetal Age (day)</th>
<th>Genotype</th>
<th>Find (lobes)</th>
<th>Organ Culture (days)</th>
<th>Cell Numbers per Lobe</th>
<th>CD4/CD8/TCR-β Three-Color Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>+/+</td>
<td>2</td>
<td>14</td>
<td>2.0 × 10⁶</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>+/-</td>
<td>2</td>
<td>14</td>
<td>2.4 × 10⁶</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>+/-</td>
<td>0</td>
<td>14</td>
<td>4.8 × 10⁵</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>+/-/-</td>
<td>2</td>
<td>14</td>
<td>0.9 × 10⁵</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>+/-</td>
<td>0</td>
<td>14</td>
<td>1.4 × 10⁵</td>
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<td>6</td>
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<td>2</td>
<td>14</td>
<td>7.4 – 12.3 × 10⁵</td>
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<tr>
<td>7</td>
<td>+/-</td>
<td>2</td>
<td>14</td>
<td>4.4 × 10⁵</td>
<td>Normal</td>
</tr>
<tr>
<td>8</td>
<td>+/-</td>
<td>0</td>
<td>14</td>
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<td>11</td>
<td>+/-</td>
<td>0</td>
<td>14</td>
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</tbody>
</table>

* Fetuses from the mating between HES-1+/− mice were individually screened for HES-1 genotype (13). Numbers of thymus lobes found are listed. At day 12.5 of gestation, thymus lobes could not be always found even in the fetuses of normal genotype (no. 3 and 5 fetuses). Thymus lobes were cultured in organ for indicated days and measured for viable cell numbers by trypan blue dye exclusion and for CD4, CD8, and TCR-β cytophocytes among all experimental groups (see Fig. 6B).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cytotypes</th>
<th>Surface markers</th>
<th>TCR-β</th>
<th>CD4/CD8</th>
<th>TCR-β</th>
<th>b</th>
<th>b</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>HES-1+/−</td>
<td>CD8+</td>
<td>CD4 TCR-β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES-1−/−</td>
<td>CD8−</td>
<td>CD2 TCR-β</td>
<td></td>
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</table>

26). Retroviral coexpression of GFP along with HES-1 allowed the detection and purification of gene-transferred cells (25). Fig. 7, A and B, shows that HES-1 expression levels markedly increased in the cells that had been infected with this HES-1-expressing retrovirus. Introduced HES-1 indeed exhibited the regulatory activity to the transcription at the HES-1 promoter (Fig. 7C).

Day 14 fetal thymocytes were infected either with HES-1-expressing virus or control GFP-expressing virus. Virus-infected thymocytes identified as GFP−CD45+ were purified using a cell sorter. Equal numbers of sorted GFP+ cells were transferred into 2-deoxyguanosine-treated fetal thymus lobes and were organ-cultured for the indicated days. It was found that thymocyte cellularity in FTOC was significantly enhanced in HES-1-introduced thymocytes than in control GFP-introduced thymocytes (Fig. 7D). CD4/CD8 phenotype and TCR-αβ/TCR-γδ ratio of thymocytes were not affected by HES-1 gene transfer (Fig. 7, E and F).

These results indicate that HES-1 overexpression in developing thymocytes increased their cell numbers, supporting the possibility that HES-1 expression level is involved in supporting the cellularity of developing thymocytes.

**Discussion**

The present results indicate that Pref-1, a member of the Delta/Notch family, is expressed in a developmentally regulated manner by TE cells. A role for interactions mediated by Pref-1 in thymocyte development is indicated by our finding that addition of dimeric Pref-1 recombinant protein to FTOC increased cellularity and that the cellularity was reduced by the addition of either a monomeric Pref-1 recombinant protein or a neutralizing anti-Pref-1 mAb. In addition to increasing the cellularity of developing thymocytes, the dimeric Pref-1 protein also stimulated increased thymocyte expression of HES-1 transcription factor.

The selective Pref-1-mediated up-regulation of HES-1 expression among the transcription factors assayed suggests that elevated levels of HES-1 might be involved in the mechanism supporting increased thymocyte cellularity in FTOC. We have described two sets of experimental data that are consistent with this possibility. First, thymocyte cellularity is consistently elevated in FTOC following retrovirus-mediated overexpression in immature thymocytes. The lineage pattern of thymocytes under these conditions was identical to that observed following administration of exogenous dimeric Pref-1 to FTOC. The second line of supportive evidence comes from analyses of thymuses from HES-1-deficient mice, where thymus cellularity was dramatically reduced and accompanied by qualitatively normal thymocyte development. The hypocellularity of HES-1-deficient FTOC and the inability of dimeric Pref-1-fusion protein to augment thymocyte cellularity in these cultures is significant in several respects. First, these data...
provide additional support for the notion that levels of HES-1 expression may represent an important regulator supporting thymocyte cellularity. In addition, the inability of Pref-1 to enhance cellularity in HES-1-deficient FTOC indicates that Pref-1 exerts its growth-promoting effects through a HES-1-dependent mechanism. While we favor the interpretation that these results reflect a mechanism whereby Pref-1-mediated interactions directly affect levels of HES-1 expression, we cannot presently exclude the possibility that the action of Pref-1 is indirect, involving other molecules but requiring HES-1 expression.

The present study shows that Pref-1 and HES-1 are both involved in supporting a number of developing thymocytes. It has been suggested that the DSL motif in the extracellular region of Delta-family molecules is crucial for the interaction with Notch-family receptor molecules (9). Because Pref-1 lacks the DSL motif, it has been speculated that Pref-1 might function through a receptor independent of Notch-family molecules, and thus independent of HES-family-mediated downstream signals. However, our results show that Pref-1 markedly up-regulates the expression of HES-1 in developing thymocytes, and that HES-1-deficient thymocytes do not respond to Pref-1, suggesting that Pref-1 affects immature thymocytes via signals through the HES-1 transcription factor. Still, it is not clear yet how Pref-1 up-regulates HES-1 expression in developing thymocytes. It is possible that immature thymocytes express the Pref-1 receptor, which directly transmits the Pref-1 binding signal to enhance HES-1 expression. However, Pref-1 in suspension culture did not affect the cellularity of immature thymocytes even in the presence of IL-7 (Fig. 3D). We have further found that, in such a suspension culture condition, Pref-1 did not increase HES-1 expression by immature thymocytes (M. Kaneta and Y. Takahama, unpublished observation). Thus, Pref-1 expressed by TE cells may affect other cells, such as mesenchymal stromal cells, which in turn produce other factors inducing HES-1 expression by thymocytes. Alternatively, Pref-1 interaction with its receptor on thymocytes may support HES-1 expression in thymocytes only in the presence of other molecular interactions between epithelial cells and thymocytes, interactions that are lost in suspension cultures. To better understand the molecular mechanism for Pref-1-induced nourishment of developing thymocytes, we aim to identify the molecular nature of the Pref-1 receptor.

Immunohistochemical analysis (Fig. 1, C and D) and RT-PCR analysis (Fig. 2B) showed that Pref-1 is in the thymus is expressed at higher levels during fetal development than in adult life. We think that the decrease in Pref-1 expression along the ontogeny is well coordinated with the function of Pref-1 presented in this study, thus supporting thymocyte numbers, because thymocyte numbers rigorously increase during early ontogeny but not in adulthood. Whether or not Pref-1 is also involved in T lymphocyte generation...
that is maintained in adult thymus (28) and that is crucial for adult T-lymphopoiesis after chemotherapy or radiation exposure would be an issue of interest and of clinical importance.

Our results show that neither Pref-1 nor HES-1 is involved in developmental choice between CD4 and CD8 T cell subsets and between TCR-αβ and TCR-γδ T cell lineages. On the other hand, Robey and colleagues (29, 30) have shown that Notch-1 is involved in these lineage decisions rather than affecting the cellularity of thymocytes. These results suggest a possibility that Notch-1 signal and HES-1 signal are not always identical, at least in developing T cells. This possibility is supported by the recent results showing that the Notch signal is capable of initiating CD4 silencer function even in the absence of a functional HES-1 binding site, although Notch signal and HES-1 signal can both repress CD4 gene expression (31). More compatible with our results are recently published results showing that Notch-1 signals are capable of rendering thymomas resistant to glucocorticoid-induced apoptosis (32), and that Notch-1 signals protect TCR-induced apoptosis (33), which suggest that the Notch-HES signaling cascade is likely involved in supporting survival of immature thymocytes.

It should be mentioned that lineage patterns of thymocytes including CD4/CD8 phenotype and TCR-αβ/TCR-γδ ratio were essentially normal either by blocking Pref-1 interactions with anti-Pref-1 Ab or in absence of HES-1 in HES-1-deficient FTOC, suggesting that the signals through Pref-1 and HES-1 equally regulate cell numbers of every thymocyte lineage, possibly by affecting cellular survival at an early CD4+CD8− stage of T cell development before the division of cell lineages. Although thymocyte cellularity is also critically regulated by IL-7, the disruption of IL-7 signals more severely reduces TCR-γδ cells than TCR-αβ cells during thymocyte development (34, 35), suggesting that the Pref-1/HES-1 signal might regulate the cellularity of thymocyte lineages in a manner more distinct than the IL-7 signal. Further analysis of the relationship between these two signal pathways as well as Bcl-2-family-mediated anti-apoptotic signals, for example antagonizing TCR signals or steroid signals, would be of great interest toward better understanding of the mechanism for growth/survival signals regulating thymocyte development.

It has been reported recently that the deficiency of either HES-1 or Notch-1 in hematopoietic precursor cells results in the arrest of T cell development at early CD4+CD8− thymocytes (36, 37), suggesting that a signaling cascade through Notch-1 and HES-1 is essential for the specification of lymphoid precursors into the T cell lineage. These results appear contradictory with our results which show hypocellularity but normal lineage distribution of HES-1-deficient thymocytes in FTOC (Table I and Fig. 6). However, the developmental arrest of thymocytes in these reports (36, 37) was observed when examining in vivo transfer of lymphopoietic precursor cells from either HES-1-deficient or Notch-1-deficient mice, unlike our results obtained from organ cultures of intact thymus lobes from HES-1-deficient mice. It is possible that the deficiency in HES-1 or Notch-1 could cause low efficiency of T-precursor cells in either migration into the thymus or early survival in the thymus. Consistent with this possibility, ex vivo analysis of Notch-1-deficient thymocytes showed hypocellularity but normal T cell lineage distribution (37). In addition, normal lineage distribution of thymocytes were generated in irradiated mice transferred with higher numbers of HES-1-deficient fetal liver cells (Dr. N. Minato, unpublished data). Consequently, we think that HES-1-mediated and perhaps Notch-1-mediated signals are critical for early survival and/or expansion of immature T-precursor cells, rather than essential for lineage specification into T lymphocytes.

Finally, our results show that the addition of a Pref-1 monomer decreased the cellularity of thymocytes, unlike Pref-1-Fc dimer proteins that increased thymocytes. We think that this decrease is due to the competitive block of endogenous Pref-1 by the tailless exogenous monomer protein. Indeed, like the Pref-1 monomer, the anti-Pref-1 mAb exhibited the similar activity in FTOC, decreasing thymocyte cellularity, thus supporting the interpretation that competitive inhibition of Pref-1 in the thymus cancels the function of Pref-1 supporting developing thymocytes.

In conclusion, the present results identify a role for Pref-1 in supporting developing T lymphocytes in the thymus. However, Pref-1 stimulation could not directly support the survival of immature thymocytes even in the presence of IL-7 (Fig. 3D), suggesting that Pref-1 and IL-7 signals are not sufficient for fully nourishing the cellularity of developing thymocytes. Attempts to identify further molecular signals supporting developing T cells are currently under the investigation.

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